A NOVEL L-THREONINE IMPORTER FROM CORYNEBACTERIUM AND A PREPARATION METHOD OF A STRAIN PRODUCING L-THREONINE

[Technical Field]

The present invention relates to a preparation method of an L-threonine producing strain by utilizing a novel L-threonine importer identified from *Corynebacterium glutamicum*.

[Background Art]

Traditional preparation methods of an amino acid-producing strain include increasing the amount of a gene expressed on the biosynthetic pathway of an objective amino acid, releasing feedback inhibition and transcription inhibition by an object project, and increasing the supply of precursor by intensifying a gene on the central metabolic pathway. In other words, the traditional breeding method was focused mainly on the cultivation of a strain whose synthesis is not easily inhibited by an excessive production of objective amino acid in a cell.

In recent years, however, active studies have been performed on various amino acid importers/exporters for use in the preparation of an amino acid-producing strain. The studies are aimed to protect many enzymes on the biosynthetic pathway from the feedback inhibition and the transcription inhibition by an object product. This is made it possible by reducing the concentration of a particular amino acid in a cell through the importer defect or the intensification of the exporter of that particular amino acid. For instance, the report on lysine exporter (*lysE*) of *Corynebacterium glutamicum* (*Microbiology*, 147:1765, 2001), and the report on the threonine production improvement by expressing threonine exporter (*thrE*) of *Corynebacterium glutamicum* from *E.coli* (*Appl. Microbiol. Biotechnol.*,

59:205, 2002) are some of the examples of the intensification of the exporter to increase the yield of a specific amino acid. As mentioned the above, the importer of a specific amino acid can also be defected to increase the yield of the amino acid. For example, the yield of tryptophan was increased by a mutant strain defective in the importer of an aromatic amino acid of *Corynebacterium glutamicum* (*Biosci, Botech. Biochem.*, 59:1600, 1995), and a strain defective in the threonine importer was prepared from *E.coli* to increase the yield of threonine (*Biosci. Botech. Biochem.*, 61:1877, 1997).

In this light, the present inventors have tried to prepare a threonine-producing strain from *Corynebacterium glutamicum* based on the discovery that the concentration of intracellular threonine was reduced and the feedback inhibition and the transcription inhibition by threonine of a threonine biosynthetic gene could be prevented by blocking transfection of threonine of high concentration into a cell, which was made it possible by defecting the threonine import pathway. That is, a threonine importer was identified and defected to produce the threonine-producing strain from *Corynebacterium glutamicum*.

[Description of the Drawings]

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

Fig. 1 illustrates a gene arrangement in a cloned DNA fragment; and

Fig. 2 illustrates a gene deficit caused by a single cross-over using an *E.coli* vector.

[Disclosure]

[Technical Problem]

Therefore, the present invention has been made in view of the above problems, and it is an object of the present invention to identify a novel L-threonine importer from *Corynebacterium glutamicum*.

It is another object of the present invention to provide a preparation method of an L-threonine producing strain defective in the importer and thus, to increase the yield of L-threonine.

[Technical Solution]

In accordance with an aspect of the present invention, the above and other objects can be accomplished by cloning and identifying a novel L-threonine importer from a wild *Corynebacterium glutamicum* strain.

In accordance with another aspect of the present invention, there is provided a preparation method of a strain defective in L-threonine importer of *Corynebacterium glutamicum*, thereby increasing the yield of L-threonine.

Although the preferred embodiments of the present invention have been disclosed for illustrative purposes, those skilled in the art will appreciate that various modifications, additions and substitutions are possible, without departing from the scope and spirit of the invention as disclosed in the accompanying claims.

[Advantageous Effects]

Therefore, the present invention can be advantageously used for preparing a threonine-producing strain from *Corynebacterium glutamicum*.

[Best Mode for Invention]

A threonine importer-defective strain was prepared from *Corynebacterium glutamicum*, in which the defective strain was used as a host strain for cloning a threonine importer.

To this end, the present inventors decided to prepare a high-threonine-requiring strain from a low-threonine-requiring strain. This was based on the assumption that if the threonine importer of the low-threonine-requiring strain was defective, it might be possible to obtain a high-threonine-requiring character. Therefore, the high-threonine-requiring strain prepared from the low-threonine-requiring strain would be the threonine importer-defective strain.

To prepare the strain, *Corynebacterium glutamicum* CJ L-1, the threonine auxotrophic strain prepared by using *Corynebacterium glutamicum* ATCC 13032, was used as a parent strain. The CJ L-1 strain demonstrated 20mg/l of auxotrophy with respect to threonine. The high-threonine-requiring strain, namely *Corynebacterium glutamicum* CJ L-11 strain, manifesting 500mg/l of auxotrophy was prepared from the CJ L-1 strain through artificial mutation. By using the CJ L-11 strain as a host strain, the genomic library of ATCC 13032 (which is the wild strain of *Corynebacterium glutamicum*) went through transformation and as a result, a low-threonine-requiring clone was obtained.

Thusly obtained clone was retransformed to the high-threonine-requiring strain to make sure that the concentration of threonine in the strain is low. Afterwards, DNA base sequence was analyzed to check which genes were contained in the cloned DNA fragment. It was discovered that the cloned DNA fragment contained 4,846 bases. Then the open reading frame (ORF) of the gene in the DNA fragment was searched again by means of the ORF Finder. In result, a predominant membrane protein gene of 1,254bp (% length) was searched. Homologous genes thereof were then searched by means of BLASTP, and it turned out that the gene manifested 48% of homology with serine/threonine transporter of *Porphyromonas gingivalis*, and 51% of homology with Na^+/H^+-dicarboxylate symporter of *Bacterioides thetaiotaomicron*. According to the report by Eikmanns *et. al.* (*Arch. Microbiol.*, 165:48, 1996), as far as *Corynebacterium glutamicum* is

concerned threonine and Na^+ are introduced into a cell at the same time, and threonine and serine are imported by a common importer. Based on these known facts, the present inventors assumed that the gene product of the cloned membrane protein in the DNA fragment could be the threonine importer, and named the gene product *thrY*.

To verify the assumption, the present inventors destroyed the gene in question of *Corynebacterium glutamicum* CJ L-1 strain (which is the low-threonine-requiring strain), in order to check if the low-threonine-requiring strain was transformed to the high-threonine-requiring strain. To prepare a defective strain, only the central part of the protein coding region of the gene went through DNA Polymerase Chain Reaction (PCR) and was cloned to an *E.coli* vector. The same was transformed to *Corynebacterium glutamicum* CJ L-1 strain, which is the low-threonine-requiring strain, and a single cross-over movement was performed thereon to get the defective strain. The same method was employed to prepare a *thrY* defective strain from *Corynebacterium glutamicum* CJ L-1 strain which is the low-threonine-requiring strain. It was found out that *thrY* defective strain manifested 300mg/l of high threonine auxotrophy. Based on these discoveries, it was concluded that the cloned *thrY* was indeed the threonine importer.

Further, the present inventors wanted to find out what happens to the yield of threonine when *thrY* of the threonine-producing strain of *Corynebacterium glutamicum* was really destroyed. For this experiment, CJ T-2, the threonine-producing *Corynebacterium glutamicum* recombination strain, was selected as a target strain, and the above-described method was used to defect the gene. Thusly prepared gene defective strain was named CJ T-21, and the same was actually used to produce threonine. It turned out that the yield of threonine was increased by 10% compared with the case where the parent strain thrI was used.

Example 1: Preparation of high-threonine-requiring strain from *Corynebacterium glutamicum*

A threonine importer-defective strain was prepared from *Corynebacterium glutamicum*, in order to use the defective strain as a host strain for cloning a threonine importer. To this end, a high-threonine-requiring strain was prepared from a low-threonine-requiring strain.

To prepare the high-threonine-requiring strain, *Corynebacterium glutamicum* CJ L-1, which is the threonine auxotrophic strain prepared by using *Corynebacterium glutamicum* ATCC 13032, was used as a parent strain. The CJ L-1 strain manifested 20mg/l of auxotrophy with respect to threonine. The CJ L-1 strain went through the artificial mutation process to produce the high-threonine-requiring strain. To induce the artificial mutation, N-methyl-N'-nitro-N-nitrosoguanidine (NTG), one of alkylating agents, was used. *Corynebacterium glutamicum* CJ L-1 grew in Luria-Bertani liquid medium until the mid of the logarithmic growth phase, and was suspended in citrate buffer (pH 5.5) to $10^7 \sim 10^8$ cells/ml. When the final concentration of the NTG became 1,000μg/ml, the CJ L-1 strain was placed in the concussor (or the shaker) at 30°C for 5 minutes. Later the strain was washed by potassium phosphate (pH 7.0) three times, and smeared over a minimal medium containing 2,000mg/l of threonine. Approximately 30,000 colonies grown in the medium were subject to the tooth picking process in the presence of the minimal medium containing 20mg/l of threonine, and any strains that were not grown were selected. These selected strains were checked again if they have the threonine-requiring character. A 500mg/l-threonine-requiring strain was finally selected and named *Corynebacterium glutamicum* CJ L-11. The CJ L-11 was then used as a host strain for cloning the threonine importer.

[Table 1]

Minimal agar medium

Ingredient	Content	Ingredient	Content
Glucose	10g	$CaCl_22H_2O$	0.1g
$(NH_4)_2SO_4$	2g	$Na_2B_4O_710H_2O$	80μg
Urea	2g	$(NH_4)_6MoO_{27}4H_2O$	40μg
KH_2PO_4	3.0g	$ZnSO_47H_2O$	10μg
$MgSO_47H_2O$	0.5g	$CuSO47H_2O$	300μg
$FeSO_47H_2O$	10mg	$MnCl_24H_2O$	10μg
$MnSO_45H_2O$	10mg	$FeCl_36H_2O$	1mg
Biotin	100μg	Agar	20g
Thiamine HCl	100μg	Distilled water	Per liter
pH (prior to disinfection) 7.0			

Example 2: Cloning of threonine importer

The *Corynebacterium glutamicum* CJ L-11, the high-threonine-requiring strain, prepared in Example 1 was used as a host strain to clone a threonine importer from ATCC 13032, the *Corynebacterium glutamicum* wild strain.

To this end, a chromosome library of *Corynebacterium glutamicum* ATCC 13032 was constructed and transformed to the *Corynebacterium glutamicum* CJ L-11, in order to obtain a low-threonine-requiring clone.

For the construction of the chromosome library, *Corynebacterium glutamicum* ATCC 13032 strain was cultured in Luria-Bertani medium for 16 hours to prepare a seed culture medium. 1% of the seed culture medium was then seeded in 10ml of Luria-Bertani medium containing 1% of glycine, and the strain was cultured therein for 12 hours. A mycobiant was collected from the cultured strain, and a chromosomal DNA was separated from the mycobiant by means of the Genomic DNA Kit manufactured by Qiangen Company. Later, 2μg of the chromosomal DNA was mixed with Sau3A1 restriction enzyme 0.1 unit and cultured for 1 hour at 37°C to be partially cut off. This partially-cut chromosomal

DNA was purified by 0.8% agarose gel electrophoresis into DNA fragments of 4-6kb. Finally, the gel-purified DNA fragment was introduced to the position of BamHI restriction enzyme of pECCG122 which is the *Corynebacterium* vector, to complete the chromosome library.

The chromosome library was then transformed to the *Corynebacterium glutamicum* CJ L-11 (which is the high-threonine-requiring strain), and smeared over the minimal medium containing 20mg/l of threonine. Afterwards, a plasmid DNA was extracted from colonies produced in the medium and retransformed to the *Corynebacterium glutamicum* CJ L-11. Finally, a clone that recovered the low-threonine-requiring character from the high-threonine-requiring character was selected and named pECCG-*thrY*.

Example 3: Base sequence analysis of cloned DNA fragment

To check the genes in the low-threonine-requiring clone obtained in Example 2, appropriate primers were synthesized and went through DNA sequencing to be overlapped. In this manner the base sequence of the cloned DNA fragment was determined (please refer to the SEQ. ID No. 1).

It turned out that the cloned DNA fragment was composed of 4,846 bases. Then the open reading frame (ORF) of the gene in the DNA fragment was searched again by utilizing the ORF Finder. In result, two ORF of longer than 1kb were searched. More specifically, the ORF1 (i.e., the SEQ. ID No. 1) was a 1,146bp gene from the 23rd base to the 1,168th base, and the other ORF2 was a 1,254bp gene from the 1,772nd base to the 3,025th base (please refer to Fig. 1).

Homologous genes thereof were then searched by means of BLASTP. It turned out that the ORF1 manifested high homology with genes such as hydroxylase or monooxygenase of various microorganisms. Meanwhile, the ORF2 manifested 48% of homology with serine/threonine transporter of *Porphyromonas gingivalis*,

and 51% of homology with Na^+/H^+-dicarboxylate symporter of *Bacterioides thetaiotaomicron*.

According to a published report, as far as *Corynebacterium glutamicum* is concerned threonine and Na^+ are introduced into a cell at the same time, and threonine and serine are imported by a common importer. Based on these known facts, the present inventors assumed that the gene product of the cloned membrane protein in the DNA fragment could be the threonine importer, and named the gene product *thrY*.

Example 4: Preparation of *thrY*-defective strain from low-threonine-requiring strain CJ L-1 and characteristics thereof

To confirm the involvement of the cloned *thrY* in the threonine import, the present inventors destroyed the gene in question of *Corynebacterium glutamicum* CJ L-1 strain (which is the low-threonine-requiring strain), in order to check if the low-threonine-requiring strain was transformed to the high-threonine-requiring strain.

To prepare the *thrY*-defective strain, only the central part of the protein coding region of the gene went through DNA Polymerase Chain Reaction (i.e., primar1: 5'-GACTTGTTCGGTGTTGAATCCGAGC-3', primar2: 5'-CGGTCTGATCGCCTACGGAGCAATC-3') and was cloned to an *E.coli* vector such as pCR2.1-TOPO (which is produced by Invitrogen Company). The same was transformed to *Corynebacterium glutamicum* CJ L-1 strain, which is the low-threonine-requiring strain, and a single cross-over movement was performed thereon to get the defective strain (please refer to Fig. 1 and Fig. 2). Later, it was found out that the threonine auxotrophy of *thrY* defective strain was markedly increased from 20mg/l to 300mg/l.

Based on these discoveries, the present inventors concluded that the cloned *thrY* was indeed the threonine importer.

Example 5: Preparation of *thrY*-defective strain from threonine-producing strain and production experiment of threonine

To find out how the destruction of *thrY* of the threonine-producing strain affects the yield of threonine, the present inventors conducted the following experiment.

For the experiment, CJ T-2, the threonine-producing *Corynebacterium glutamicum* recombination strain, was selected as a target strain, and the above-described method was used to defect the gene. Thusly prepared gene defective strain was named CJ T-21, and the same was actually used to produce threonine.

As for the fermentation, the strain was placed in a 250ml baffle flask (culture medium: 25ml) and cultured in a concussor (or a shaker) at 30°C and 230rpm for 72 hours. The composition of the fermentation medium is illustrated in the following Table 2. After measuring the concentration of threonine, the present inventors discovered that the parent strain accumulated 7.3g/l of threonine in the culture medium, and the thrI defective strain accumulated 8.1g/l of threonine, showing approximately 10% of increase. This is because threonine import into a cell was basically blocked because of the defective *thrY*, so the concentration of threonine in the cell was reduced. Accordingly, threonine biosynthetic genes could avoid the feedback inhibition or the transcription inhibition by threonine.

[Table 2]

Flask fermentation medium

Ingredient	Content
Molasses (reducing sugar)	100g
Yeast concentrated extract	4g
$(NH_4)_2SO_4$	40g
Urea	4g

KH_2PO_4	1g
NaCl	2.5g
$MgSO_47H_2O$	0.5g
$FeSO_47H_2O$	10mg
$MnSO_45H_2O$	10mg
Biotin	100μg
Thiamine HCl	200μg
$CaCO_4$	40g
Process water	Per liter
pH (after disinfection)	7.0